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ANTIOXIDANT ACTIVITY OF *Annona muricata* LEAVES – AN *IN VITRO* STUDY

K. Shibula and S. Velavan*

¹Research Scholar, PG and Research Department of Biochemistry, Marudupandiyar College, Tamil Nadu, S. India

² PG and Research Department of Biochemistry, Marudupandiyar College, Tamil Nadu, S. India,

*Corresponding author

ABSTRACT

Antioxidant activity of *Annona muricata* leaves extract were carried out for proving its utility in free radical mediated diseases including diabetic, cardiovascular, cancer etc. The ethanolic extract was screened for *in vitro* antioxidant activity by oxygen radical scavenging such as DPPH, total antioxidant assay, superoxide, metal chelation and iron reducing power activity at different concentrations. Throughout the studies leaves extract showed marked antioxidant activity. The antioxidant activity of the leaves extract may be due to the phytochemicals present in it. The antioxidant activity was found to be concentration dependent and may be attributed to the presence of bioflavonoids content in *Annona muricata* leaves extract (AMLE). Overall, the plant extract is a source of natural antioxidants which might be helpful in preventing the progress of various oxidative stress mediated diseases including aging.

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*Corresponding author

Dr. S. Velavan, P.G.
and Research
Department of
Biochemistry,
Marudupandiyar
College,
Tamil Nadu, India.

INTRODUCTION

In living organisms, various reactive oxygen and nitrogen species (ROS/RNS) e.g., superoxide anions ($O_2^{\cdot-}$), hydroxyl radicals ($\cdot OH$), nitric oxide radicals ($NO\cdot$) and non-radical compounds, can be formed by different mechanisms. It is unavoidable one because of they are continuously produced by the body's normal use of oxygen. Such species are considered to be important causative factors in the development of diseases such as diabetes, stroke, arteriosclerosis, cancer, and cardiovascular diseases and the aging process (Velavan, 2011; Alma *et al*, 2003). This effect was significantly reversed by prior administration of

antioxidant providing a close relationship between free radical scavenging activity (FRSA) and the involvement of endocrinological responses (Wiseman and Halliwell, 1996).

The recent abundant evidence suggesting the involvement of oxidative stress in the pathogenesis of various disorders and diseases has attracted much attention of the scientists and general public to the role of natural antioxidants in the maintenance of human health and prevention and treatment of diseases (Niki, 2010). Plant and its products are rich sources of a phytochemicals and have been found to possess a variety of biological activities including antioxidant potential (Velavan *et*

al, 2007). The majority of the active antioxidant constituents are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, and isocatechins. In addition to the above compounds found in natural foods, vitamins C and E, beta-carotene, and tocopherol are known to possess antioxidant potential (Prior, 2003). With this background and abundant source of unique active components harbored in plants. The chosen medicinal plant namely as *Annona muricata* (Tamil: Mulluchitta) leaves belongs to the Annonaceae family. Therefore, the present study were to investigate the free radical scavenging activity of *Annona muricata* leaves through the free radical scavenging such as DPPH scavenging, superoxide anion radical scavenging, metal chelation, reducing power activity and total antioxidant assay.

MATERIALS AND METHODS

Chemicals

Nitro blue tetrazolium (NBT), ethylene diamine tetra acetic acid (EDTA), sodium nitroprusside (SNP), trichloro acetic acid (TCA), thio barbituric acid (TBA), potassium hexa cyano ferrate [K₃Fe(CN)₆], and L-ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd., India. All other chemicals and solvents used were of analytical grade available commercially.

Plant materials

The fully mature *Annona muricata* leaves were collected in April 2015 from Home garden, Pattom, Thiruvendram, Kerala, India from a single herb. The leaves were identified and authenticated by Dr. S. John Britto, The Director, the Rabinat Herbarium and centre for molecular systematics, St. Joseph's college Trichy-Tamilnadu. India. A Voucher specimen has been deposited at the Rabinat Herbarium, St. Josephs College, Thiruchirappalli, Tamil nadu, India.

Preparation of alcoholic extract

The collected *Annona muricata* leaves were washed several times with distilled water to remove the traces of impurities from the leaves. The leaves were dried at room temperature and coarsely powdered. The powder was extracted with 70% methanol for 48 hours. A semi solid extract was obtained after complete elimination of alcohol under reduced pressure. The *Annona muricata* leaves extract (AMLE) was stored in refrigerator until used. Doses such as 20, 40, 60 and 80µg/ml were chosen for in vitro antioxidant activity.

DPPH ASSAY

The scavenging ability of the natural antioxidants of the plant extract towards the stable free radical DPPH was measured by the method of

Shimada *et al.* (1992). Briefly, a 2 ml aliquot of DPPH methanol solution (25µg/ml) was added to 0.5 ml sample solution at different concentrations. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. Then the absorbance was measured at 517nm in a spectrophotometer. L-Ascorbic acid was used as the standard.

Radical scavenging activity (%)

$$= 100 - \frac{A_C - A_S}{A_C} \times 100$$

Where A_C = control is the absorbance of the control and A_S = sample is the absorbance of reaction mixture (in the presence of sample). All tests were run in triplicates (n = 3), and the average values were calculated.

Determination of total antioxidant capacity

The antioxidant activity of the extracts was evaluated by the phosphomolybdenum method according to the procedure of Prieto *et al.* (1999). The assay is based on the reduction of Mo(VI)–Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. 0.3 ml extract was combined with 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Superoxide anion scavenging activity assay

The scavenging activity of the *Annona muricata* leaves towards superoxide anion radicals was measured by the method of Liu *et al.* (1997). Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 ml of Tris-HCl buffer (100mM, pH 7.4) containing 0.75 ml of NBT (300 µM) solution, 0.75 ml of NADH (936 µM) solution and 0.3 ml of different concentrations of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 µM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = [(A_0 - A_1) / A_0 \times 100],$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Fe²⁺ chelating activity assay

The chelating activity of the extracts for ferrous ions Fe²⁺ was measured according to the method of Dinis *et al.* (1994). To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of FeCl₂ (2 mM) was added. After 30 s, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe²⁺-Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe²⁺ was calculated as

$$\text{Chelating rate (\%)} = (A_0 - A_1) / A_0 \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Reducing power assay

The Fe³⁺ reducing power of the extract was determined by the method of Dinis (1994) with slight modifications. The extract (0.75 ml) at various concentrations was mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) and 0.75 ml of potassium hexacyanoferrate [K₃Fe(CN)₆] (1%, w/v), followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride (FeCl₃) solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Statistical analysis: Tests were carried out in triplicate for 3–5 separate experiments. The amount of extract needed to inhibit free radicals concentration by 50%, IC₅₀, was graphically estimated using a nonlinear regression algorithm.

RESULTS AND DISCUSSION

The search for new molecules, nowadays, has taken a slightly different route where the science of ethnobotany and ethnopharmacognosy are being used as guide to lead the chemist towards different sources and classes of compounds (Gurib-Fakim, 2006). Plant derived natural products hold great promise for discovery and development of new pharmaceuticals (McChesney *et al.*, 2007). Our earlier reports indicates the methanolic extract of *Annona muricata* leaves extract contains flavonoids, saponin, terpenoids, steroids, alkaloids, polyphenols and saponin which are an important in disease prevention and health preservation.

DPPH Assay

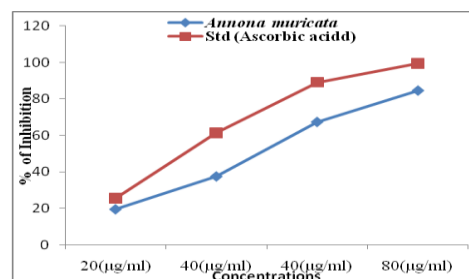
Recently, the use of the DPPH[•] reaction has been widely diffused among food technologists and researchers, for the evaluation of free radical scavenging activity on extracts from plant, food material or on single compounds. In the DPPH assay, the antioxidant was able to reduce the stable radical DPPH to the yellow colored 1, 1-diphenyl-1, 2-picryl hydrazine. The molecule of 2, 2-diphenyl-1-picryl hydrazine is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole. The proton transfer reaction of the DPPH[•] free radical by a scavenger causes a decrease in absorbance at 517 nm, which can be followed by a common spectrophotometer set in the visible region. The effect of antioxidants on DPPH[•] is thought to be due to their hydrogen donating ability (Sindhu and Abraham, 2006). DPPH radical scavenging activity of plant extract of FFLE and standard as ascorbic acid are presented in Table 1 and Fig 1. The DPPH radical was widely used to evaluate the free-radical scavenging capacity of antioxidants (Nuutila *et al.*, 2003). The half inhibition concentration (IC₅₀) of ascorbic acid and plant extract were 53.54 μg ml⁻¹ and 32.91 μg ml⁻¹ respectively. The plant extract exhibited a significant dose dependent inhibition of DPPH activity. The potential of L-ascorbic acid to scavenge DPPH radical is directly proportional to the concentration. The DPPH assay activity is near to standard as ascorbic acid.

Table 1- % of DPPH Radical scavenging activity of *Annona muricata* extract at different concentrations

Concentration (μg/ml)	<i>Annona muricata</i> Extract	Standard (Ascorbic acid)
20	19.52±2.76	25.6±2.04
40	37.51±3.32	61.26±4.90
60	67.34±4.01	88.98±7.11
80	84.53±5.21	99.34±7.94
IC ₅₀	53.54	34.91

Values were expressed as Mean ± SD for Values

Fig 1- % of DPPH Radical scavenging activity of *Annona muricata* extract at different concentrations



Total antioxidant activity

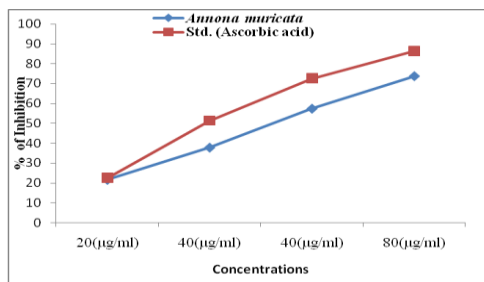
The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/ Mo (V) complex with a maximal absorption at 695 nm. The assay is successfully used to quantify vitamin E in seeds and, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant extract (Prieto *et al.*, 1999). Moreover, it is a quantitative one, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The yield of the ethanol extract of the plant extract and its total antioxidant capacity are given in Table 2 Fig 2. Total antioxidant capacity of FFLE is expressed as the number of equivalents of ascorbic acid. The study reveals that the antioxidant activity of the extract is in the increasing trend with the increasing concentration of the plant extract. The half inhibition concentration (IC₅₀) of ascorbic acid and plant extract were 45.85µg ml⁻¹ and 42.41 µg ml⁻¹ respectively.

Table 2- % of Total antioxidant activity of *Annona muricata* extract at different concentrations

Concentration (µg/ml)	<i>Annona muricata</i> Extract	Standard (Ascorbic acid)
20	21.59±2.21	22.35± 1.80
40	37.78±2.64	51.23± 4.09
60	57.34±3.31	72.54± 5.80
80	73.67±3.75	86.35± 6.91
IC ₅₀	45.85	42.41

Values were expressed as Mean ± SD for Values

Fig 2- % of Total antioxidant activity of *Annona muricata* extract at different concentrations



Superoxide anion radical scavenging activity

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system (Korycka-Dahl & Richardson, 1978). The superoxide anion radical scavenging activities of the extract from *Annona muricata*

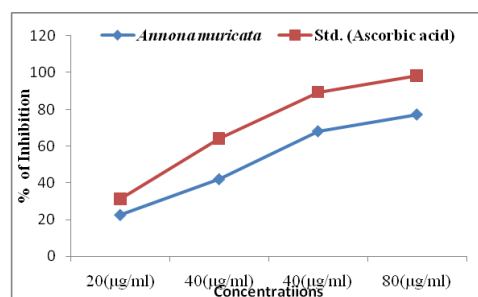
assayed by the PMS-NADH system were shown in Table 3 and Fig 3. The superoxide scavenging activity of *Annona muricata* was increased markedly with the increase of concentrations. The half inhibition concentration (IC₅₀) of *Annona muricata* was 44.84 µg ml⁻¹ and ascorbic acid were 31.62µg ml⁻¹ respectively. These results suggested that *Annona muricata* had notably superior superoxide radical scavenging effects.

Table 3- % of Superoxide Radical scavenging activity of *Annona muricata* extract at different concentrations

Values were expressed as Mean ± SD for Values

Concentration (µg/ml)	<i>Annona muricata</i> Extract	Standard (Ascorbic acid)
20	22.51±2.27	31.25 ± 2.50
40	41.99±2.58	64.23 ± 5.13
60	68.06±2.66	89.54 ± 7.16
80	77.21±3.30	98.51 ± 7.88
IC ₅₀	44.84	31.62

Fig 3- % of Superoxide Radical scavenging activity of *Annona muricata* extract at different concentrations



The ferrous ion chelating activity

Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of color reduction. The formation of the ferrozine- Fe²⁺ complex is interrupted in the presence of aqueous extract of *Annona muricata*, indicating that have chelating activity with an IC₅₀ of 48.99 µg ml⁻¹ and ascorbic acid was 30.96µg ml⁻¹ respectively (Table 4 and Fig 4). Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxy and alkoxy radicals (Halliwell, 1991; Fridovich, 1995). Metal chelating activity can contribute in reducing the concentration of the catalyzing

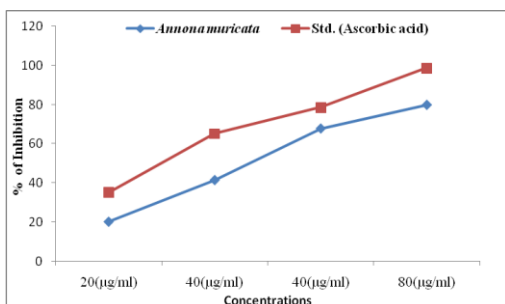
transition metal in lipid peroxidation. Furthermore, chelating agents that form s bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, and thereby stabilize the oxidized form of the metal ion (Gordon, 1990). Thus, *Annona muricata* demonstrate a marked capacity for iron binding, suggesting their ability as a peroxidation protector that relates to the iron binding capacity.

Table 4- % of Iron chelating activity of *Annona muricata* extract at different concentrations

Concentration (µg/ml)	<i>Annona muricata</i> Extract	Standard (Ascorbic acid)
20	20.22±5.96	35.23 ± 2.81
40	41.49±5.70	65.21 ± 5.28
60	67.79±6.14	78.51± 6.28
80	79.86±6.29	98.65 ± 7.89
IC ₅₀	48.99	30.96

Values were expressed as Mean ± SD for Values

Fig 4- % of Iron chelating activity of *Annona muricata* extract at different concentrations



Reducing power activity

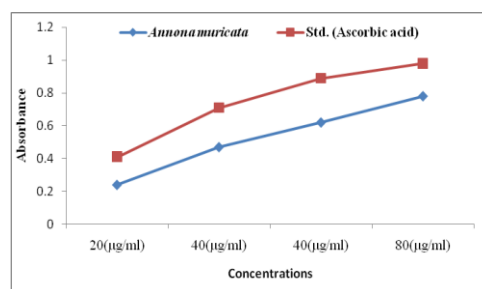
The measurements of the reducing ability, the Fe³⁺-Fe²⁺ transformation was investigated in the presence of *Annona muricata*. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997; Yildirim *et al*, 2000). Table 5 and Fig 5 depicts the reductive effect of *Annona muricata*. Similar to the antioxidant activity, the reducing power of *Annona muricata* increased with increasing dosage. All the doses showed significantly higher activities than the control exhibited greater reducing power, indicating that *Annona muricata* consist of hydrophilic polyphenolic compounds that cause the greater reducing power.

Table 5- Reducing power assay of *Annona muricata* extract at different concentrations

Concentration (µg/ml)	<i>Annona muricata</i> Extract	Standard (Ascorbic acid)
20	0.24±0.08	0.41± 0.03
40	0.47±0.08	0.71 ± 0.05
60	0.62±0.09	0.89 ±0.07
80	0.78±0.09	0.98 ± 0.08

Values were expressed as Mean ± SD for Values

Fig 5- Reducing power assay of *Annona muricata* extract at different concentrations



CONCLUSION

On the basis of the results of this study, it clearly indicates that *Annona muricata* leaves had powerful *in vitro* antioxidant capacity against various antioxidant systems as DPPH, nitric oxide, superoxide anion scavenging and metal chelator. From our results, the antioxidant activity of *Annona muricata* leaves was concentration dependent. The extracts could exhibit antioxidant properties approximately comparable to commercial synthetic antioxidants as ascorbic acid. From the above assays, the possible mechanism of antioxidant activity of *Annona muricata* leaves includes reductive ability, metal chelator, hydrogen donating ability and scavengers of superoxide and free radicals.

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